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Protein–Protein Interactions between Epstein–Barr Virus Nuclear Antigen-LP and Cellular

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The EBNA-LP protein encoded by the open reading frame in the leader exons of the Epstein–Barr nuclear antigen messages is essential for efficient immortalization of B lymphocytes. Protein–protein interaction studies using affinity precipitation of proteins from [^{35}S]methionine-labeled cell lysates and bacterially expressed maltose binding protein EBNA-LP fusions were performed. A cellular 68/72-kDa doublet protein was detected. This banding pattern was shown to be identical to that obtained in affinity precipitations with fusions of glutathione–S-transferase and Sp1 (a basal transcription factor). For both EBNA-LP and Sp1 the specific interacting cellular proteins have been identified as heat shock proteins (HSP) 72/73. Affinity precipitation of HSP 72/73 with deletion mutants of EBNA-LP maps the interaction domain on EBNA-LP to exon Y2 which is required for immortalization. Immunoprecipitation of EBNA-LP from EBV-positive lymphoblastoid cell lines coprecipitated the HSP 72/73 proteins, indicating that the interaction occurs *in vivo* as well as *in vitro.* The association of HSPs with a widening range of nuclear proteins involved in gene expression and proliferation control now includes Sp1 and EBNA-LP and suggests that there is a central role for molecular chaperones in these processes.

INTRODUCTION

EBV infects and immortalizes B lymphocytes (Leibowitz and Kieff, 1990; Klein, 1994; Thorley-Lawson and Mann, 1985). Although most infections are resolved by cell-mediated immune responses under certain conditions EBV immortalized B cells can progress to a malignant lymphoma (Khanna *et al.,* 1995; Klein, 1983; Penn *et al.,* 1969). Genetic analyses of the virus have demonstrated roles for several viral genes (LMP1, EBNA1, 2, 3A, 3C, and 4) in the immortalization process (Robertson and Kieff, 1995). LMP1 (latent membrane protein 1) is an integral membrane protein and may be a growth factor receptor but certainly plays some role in signal transduction since it interacts with TRAF1 (tumor necrosis factor receptor associated factor 1) and LAP1 (LMP associated protein 1), a RING-finger-motif-bearing protein with considerable homology to TRAF2 (Mosialos *et al.,* 1995). EBNA2 and 3C are transcriptional activators (Marshall and Sample, 1995; Tsui and Schubach, 1994). EBNA2, in particular, activates expression of the other EBNAs, including EBNA3C and the LMP1 protein by activating EBNA2 response elements in the upstream regions of responsive promoters. EBNA2 can be found complexed with a cellular protein RBP-J_K at the conserved core sequence of the EBNA2 response elements (Zimber-Strobl *et al.,* 1994; Ling *et al.,* 1993; Matsunami *et al.,* 1989; Henkel *et al.,* 1994; Waltzer *et al.,* 1994; Leibowitz and Kieff, 1990). Cellular genes for the B cell activation markers CD21 and CD23 and the oncogene c-fgr are also activated in EBNA2 expressing cells (Cordier-Bussat *et al.,* 1993). EBNA1 is a viral origin of replication (ori P) binding protein which is essential for maintenance of the viral episome in proliferating cells (Rawlins *et al.,* 1985). The roles of the other proteins in the immortalization process (EBNA3B, EBNA-LP, and LMP2) are not well characterized.

The open reading frame for EBNA-LP is located in the leader exons of the spliced mRNAs of the EBNA transcription unit. It is derived from the repeating W1 and W2 exons of the major internal repeat (IR-1) and the COOH terminal Y1 and Y2 exons. The length of the W1W2 reiteration varies in different cDNAs and leads to expression of a series of related protein species in virus-infected cells. The minimal EBNA-LP polypeptide with only one repeat would be 110 residues in length and rich in argenines (19 residues) and prolines (18 residues). Recombinant viruses lacking Y1 and Y2 exons are defective, but not entirely deficient, in the immortalization phenotype (Mannick *et al.,* 1991; Hammerschmidt and Sugden, 1989). Immortalization efficiency is reduced 10-fold and requires the presence of fibroblast feeder layers. Immortalized cells grow slowly with an atypical small rounded appearance. With passage in culture cells eventually achieve lymphoblastoid features and feeder layer independence; however, most colonies do not give rise to long-term lymphoblastoid cell lines.

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EBNA-LP has been reported to colocalize by immunofluorescence in the nucleus of infected cells with Rb (the retinoblastoma susceptibility gene product) (Jiang *et al.,* 1991). In addition, Rb and the tumor suppressor p53 were both found to bind to EBNA-LP in *in vitro* binding experiments. The region of EBNA-LP involved in binding was the argenine-rich segment encoded by the W1 exon of the repeating W1W2 domain (Szekely *et al.,* 1993). These findings have led to the suggestion that EBNA-LP function may be analogous to that of nuclear oncogene proteins encoded by other viruses and involve impairment of Rb and p53. Recently, however, it was shown that immortalized lymphoblasts treated with DNA damaging agents are capable of undergoing p53-mediated apoptosis, indicating that p53 function is not neutralized by the expression of EBNA-LP (Allday *et al.,* 1995).

Previous work has focused attention on cell-cycle-related effects of EBNA-LP expression (Allan *et al.,* 1992). Along with EBNA2, it is the first viral gene product detected upon primary infection of resting lymphoblasts and EBNA-LP mutant virus immortalized cells show delayed transit through the G1 phase of the cell cycle. Resting B cells, primed by CD21 crosslinking with the gp340 virus envelope protein to mimic virus infection and transfected with EBNA2 and EBNA-LP expression vectors, enter and progress through the cell cycle as evidenced by synthesis of cyclin D2 (Sinclair *et al.,* 1994). The conclusion of these studies is that EBNA2 and EBNA-LP are key regulators of cyclin D2 expression and that these two viral proteins cooperate to induce a G0 to G1 transition.

In this report we have examined the ability of EBNA-LP to interact with cellular proteins in extracts of cells metabolically labeled with [^{35}S]methionine. A p68 72-kDa doublet interacting species was detected and has subsequently been identified as human heat shock protein (HSP). The same proteins are also shown to interact with the basal transcription factor Sp1 and, as previously reported, the Rb retinoblastoma gene product (Nihei *et al.,* 1993). The domains of EBNA-LP required for immortalization are also responsible for HSP interaction.

MATERIALS AND METHODS

Cells

X50-7 cells are human cord lymphocytes immortalized *in vitro* and express only the genes for EBNAs and LMPs (Wilson and Miller, 1979). IARC/BL36 is an EBV-positive Burkitt's lymphoma cell line kindly provided by G. Lenoir (Lenoir *et al.,* 1985). DG-75 cells are an EBV-negative Burkitt's lymphoma cell line (Ben-Basset *et al.,* 1977) morphologically similar to EBV immortalized B cells. 293 cells are a human embryonic kidney cell line transformed by adenovirus DNA and expressing E1A and E1B gene products (Graham *et al.,* 1977). 293 cells were grown in Dulbecco's modified Eagle's medium supplemented with 2 m*M* glutamine, 100 IU of penicillin, and 100 μg of streptomycin per milliliter (Gibco BRL, Gaithersburg, MD) and 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA). The lymphocytic cell lines were grown in RPMI 1640 with Glutamax (Gibco BRL) supplemented with 100 IU penicillin, 100 μg streptomycin per milliliter, and 10% fetal bovine serum. All cells were grown at 37° in a humidified 5% CO_2 atmosphere.

Antibodies

The monoclonal clone PMG3-245 directed against Rb and against clone M58 adenovirus type 5 E1a as well as rabbit anti-mouse IgG1 were obtained from Pharmingen (San Diego, CA). The monoclonal antibody to the EBNA-LP protein, JF186 (Finke *et al.,* 1987), was kindly provided by M. Rowe (University of Birmingham, UK). The mouse monoclonal antibody clone N27F3-4 specific for constitutive and inducible forms of Hsp 70 (Hsc 73 and Hsp 72) and rat monoclonal antibody clone 1B5 specific for constitutive Hsc 73 as well as purified recombinant human Hsp 70 protein were obtained from Stress-Gen Biotechnologies (Victoria, BC, Canada). The polyclonal antibody to maltose binding protein (MBP) was obtained from New England Biolabs (Beverly, MA). Rabbit anti-rat IgG was obtained from Sigma (St. Louis, MO).

Plasmids

The pGEX-2T plasmid encloding the leader sequences of glutathione–S-transferase (GST) (Pharmacia, Piscataway, NJ) was a gift from P. Robbins (University of Pittsburgh). The GST–Rb plasmid containing GST fused to a fragment corresponding to amino acids 379–928 of wild-type Rb were provided by W. Kaelin (Dana Farber Cancer Institute). The pGEX-E1a plasmid carrying GST fused in frame to the E1a gene was provided by P. Branton (McGill University, Montreal, Canada). The GST–SP1a plasmid containing GST fused to the SP1 gene was a gift from J. Horowitz (Duke University). The plasmid pMAL-cRI which encodes an MBP–β-galactosidase fusion protein (MBP–lacZ) was purchased from New England Biolabs. The plasmid pMBP was constructed by inserting a stop codon downstream of the ma/E gene on pMAL-cRI, thereby inhibiting expression of lacZ. Plasmids encoding the MBP fused to a one-repeat EBNA-LP (pMBP-W1W2Y1Y2) or various fragments of EBNA-LP (pMBP-W1, pMBP-W1W2, pMBP-W1W2Y1, pMBP-W2Y1Y2, pMBP-Y1Y2, and pMBP-Y2) were obtained by PCR amplification of a one-repeat EBNA-LP cDNA clone, pSp64-WY1 (derived from pIB4WY kindly provided by S. Speck, St. Louis). Each 5′ amplimer contained an *Eco*RI site and each 3′ amplimer contained either a *Bam*HI site or an *Xba*I site such that the resulting PCR product, upon digestion with the *Eco*RI and *Bam*HI or *Eco*RI and *Xba*I, could be ligated in frame downstream of the malE gene of pMAL-cRI. All clones were verified by restriction mapping and DNA

sequencing using a Sequenase 2.0 kit (U.S. Biochemical Corp.) using the protocol suggested by the manufacturer.

Preparation of bacterial fusion proteins

MBP and glutathione–S-transferase fusion protein expression and purification were essentially as described by Smith and Johnson (1988). For preparation of fusion proteins to be used as substrates in affinity precipitation experiments, overnight cultures of *Escherichia coli* (INVαF′; Invitrogen) transformed with plasmid DNA were diluted 1:10 in Luria–Bertani medium (LB) containing ampicillin (100 μg/ml) and incubated at 37° for 2 hr. Fusion proteins were induced by addition of 0.2 m*M* isopropylthioglycoside (IPTG) to the culture for 3 hr after which cells were recovered by centrifugation (5000 *g* for 15 min) at 4° and lysed on ice by sonication with two 30-sec pulses in a 1/10 volume of NETN buffer [20 m*M* Tris–Cl (pH 8.0), 100 m*M* NaCl, 1 m*M* EDTA, 0.5% NP-40]. The suspension was cleared by centrifugation at 12,000 *g* for 15 min at 4°. Cleared lysates were mixed with either 50 μl glutathione–Sepharose 4B (Pharmacia, Piscataway, NJ) or amylose agarose (New England Biolabs) per 1 ml of lysate and incubated for 1 hr at 4°. Beads were washed three times with NETN buffer and blocked with NETN containing 5% nonfat milk. For analysis of concentration and purity of bound proteins, beads were boiled in 2× SDS sample buffer and loaded onto SDS–polyacrylamide gels. Proteins were visualized by Coomassie blue staining in comparison to protein standards of known concentration.

Large-scale preparation of purified MBP–W1W2Y1Y2 for *in vitro* binding assays was essentially as described by the protocol of New England Biolabs. Cells transformed with pMBPW1W2Y1Y2 were grown in 1 liter of LB medium containing 0.2% glucose and 100 μg/ml ampicillin to an optical density at 600 nm of 0.6. IPTG was added to a final concentration of 0.3 m*M.* After 2 hr at 37°, the cells were harvested by centrifugation, and the pellet was resuspended in 50 ml of column buffer [20 m*M* Tris–Cl (pH 7.4), 200 m*M* NaCl, 1 m*M* EDTA]. The suspension was sonicated at 4° for five cycles of 1 min each and then centrifuged at 12,000 *g* for 15 min at 4°. The cleared supernatant was loaded onto a 5-ml column of amylose resin (New England Biolabs) equilibrated in column buffer and washed with 10 column volumes of column buffer. The purified protein was eluted with column buffer + 10 m*M* maltose, and fractions were analyzed by SDS–PAGE followed by Coomassie staining.

Metabolic labeling and cell lysis

Metabolic labeling was essentially as described by Kaelin *et al.* (1991). Exponentially growing DG-75 cells were fed fresh media 12–18 hr prior to radioisotopic labeling. The following day, cells were pelleted by centrifugation and washed once with Tris-buffered saline (TBS) [25 m*M* Tris–Cl (pH 8.0), 150 m*M* NaCl]. The cells were resuspended in methionine-free RPMI at 1×10^7 cells/ml and incubated at 37° for 1 hr. The medium was supplemented with a mixture of [^{35}S]-methionine and [^{35}S]cysteine (100 μCi/ml medium: Tran ^{35}S-label, 100 Ci/mmol, ICN Biomedicals, Inc.), 1 m*M* glutamine, and 5% dialyzed fetal bovine serum. The cells were incubated for 4 hr and then harvested by centrifugation, washed twice with ice-cold TBS, and lysed for 1–2 hr in ice-cold EBC buffer [50 m*M* Tris–Cl (pH 8.0), 120 m*M* NaCl, 0.5% NP-40, 10 m*M* NaF, 200 μ*M* sodium orthovanadate] containing 10 μg/ml of the protease inhibitors aprotinin, phenylmethylsulfonyl fluoride, and leupeptin. The lysates were cleared by centrifugation at 14,000 *g* for 15 min at 4°.

Affinity precipitation and *in vitro* binding assay

GST fusion proteins were expressed in bacteria and recovered on gluathione beads. MBP fusion proteins were expressed and recovered on amylose agarose beads. The beads loaded with fusion proteins were rocked with aliquots of precleared whole-cell lysates representing 5×10^6 cells. Lysates were precleared by incubating with either glutathione–Sepharose preloaded with the GST protein or amylose–agarose preloaded with the MBP protein for 1 hr at 4°. After 1 hr of affinity binding of cell proteins from the lysate the beads were washed three times with NETN and resuspended in 2× SDS sample buffer [0.625 *M* Tris (pH 6.8), 2% SDS, 0.15 2-mercaptoethanol, 10% glycerol, 0.02 m*M* bromphenol blue] and boiled. Bead-bound proteins were resolved by SDS–PAGE and visualized by fluorography or immunoblotting. EN^{3}HANCE (Dupont) was used as a secondary fluor to increase sensitivity.

Direct binding of purified human recombinant Hsp 70 (Stress-Gen Biotechnologies) to MBP–EBNA-LP fusion proteins and MBP–W1W2Y1Y2 to GST–Rb was performed in NTN buffer (NETN buffer without EDTA). Purified Hsp 70 (2 μg) and MBP–W1W2Y1Y2 (5 μg) in 250 μl NTN were combined with 50 μl of bead-bound unblocked MBP–EBNA-LP fusion protein derivatives or GST–Rb and incubated at 4° for 2 hr with rocking. The beads were washed three times with NTN, denatured in gel sample buffer, and separated by SDS–PAGE. Bound proteins were detected by immunoblotting with the appropriate antibody.

Immunoprecipitation and immunoblotting

Extracts from the equivalent of 2×10^7 cells were twice precleared for 1 hr each time with protein A–Sepharose beads (Sigma) equilibrated in NETN. Precleared supernatants were used for immunoprecipitation overnight at 4° with protein A–Sepharose beads precoated with saturating amounts of the indicated antibodies. When mAbs were used, protein A–Sepharose was loaded with rabbit

anti-mouse IgG1 (Pharmingen) or rabbit anti-rat IgG (Sigma) to provide an isotype-specific affinity matrix. Denatured immune complexes were separated by electrophoresis, transferred to Immobilin PVDF membranes (Millipore, Bedford, MA), and blocked with Tris–saline (10 m*M* Tris, pH 8.0, 150 m*M* NaCl) containing 5% nonfat milk. Monoclonal and polyclonal antibodies at appropriate dilutions were reacted at 4° from 4 hr to overnight. Goat α-mouse ^{125}I (ICN Biomedicals, Inc., 2–15 μCi/μg) or protein A ^{125}I (ICN Biomedicals, Inc., 70–100 μCi/μg) was used to visualize sites of antibody binding. Detection of EBNA-LP–Hsp complexes was achieved by immunoprecipitation with one antiserum (e.g., anti-EBNA-LP) and immunoblotting with antisera to the other (e.g., anti-Hsp) or vice versa.

RESULTS

Affinity precipitation of ^{35}S-labeled proteins from lymphocytes

The EBNA-LP protein is probably similar to other viral nuclear oncoproteins in that it affects cell growth by making physical associations with cellular proteins. In other systems these interactions have been initially studied by affinity precipitation using bacterial fusion proteins as lures (Kaelin *et al.,* 1991). We constructed a series of MBP fusions containing one to seven W1W2 repeats and Y1 and Y2 exons for these experiments. Fusions containing more than two repeats were highly unstable and degraded during synthesis in bacteria (not shown). Even two repeat containing fusion proteins were not recoverable in high yield; therefore, most of the affinity precipitation studies have been done with the more stable one repeat containing fusion proteins. The contribution of multiple copies of repeats to affinity binding of cellular proteins cannot be addressed with bacterially expressed fusion proteins.

The undifferentiated EBV-negative B lymphoma cell line DG75 was labeled with [^{35}S]methionine for 4 hr and a whole cell lysate was prepared. After clarification by low-speed centrifugation to remove insoluble debris, the lysate was precleared by incubation with MBP bound to amylose–agarose beads to remove nonspecific binding proteins. Aliquots of precleared lysate were then incubated with amylose beads loaded with MBP or MBP–W1W2Y1Y2. Eluted cellular proteins were separated on polyacrylamide gels and detected by autoradiography. After rinsing unbound or loosely bound cellular proteins away, specific binding proteins were eluted with 10 m*M* maltose. Two proteins initially designated p68 and p72 based on their apparent molecular weights were specifically bound to EBNA-LP fusions (Fig. 1). In different experiments the p68 species often appeared to be the more intense species. In cell fractionation studies there was a marked tendency to find more protein in nuclear pellets than cytoplasmic extracts; however, there was no clear

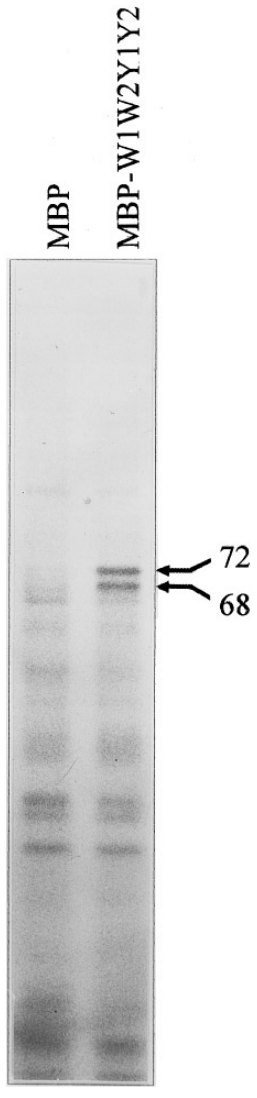

FIG. 1. Affinity binding of ^{35}S-labeled cellular proteins from the EBV-negative BL cell line DG-75 to bacterial fusion proteins. Purified maltose binding proteins (MBP) or MBP fused to the minimal coding sequence for EBNA-LP were attached to amylose beads and used as lures for protein–protein interactions.

localization or greatly enhanced recovery of interacting proteins from different cellular compartments. The close migration rates of the two proteins and the variation in relative intensity suggested that these may be isoforms of the same polypeptide product. Occasionally a protein of 105 kDa molecular weight was also detected, but only with eluates subjected to no washes or one wash with very mild (detergentless) conditions. Suspecting that this was the previously reported Rb association, affinity precipitates of this type were immunoblotted and probed for the presence of Rb (Szekely *et al.,* 1993). The Rb protein was never detected in these or any of our affinity precipitation experiments which have included cell fractionation procedures and variation of binding conditions (pH and mono- and divalent cation concentrations).

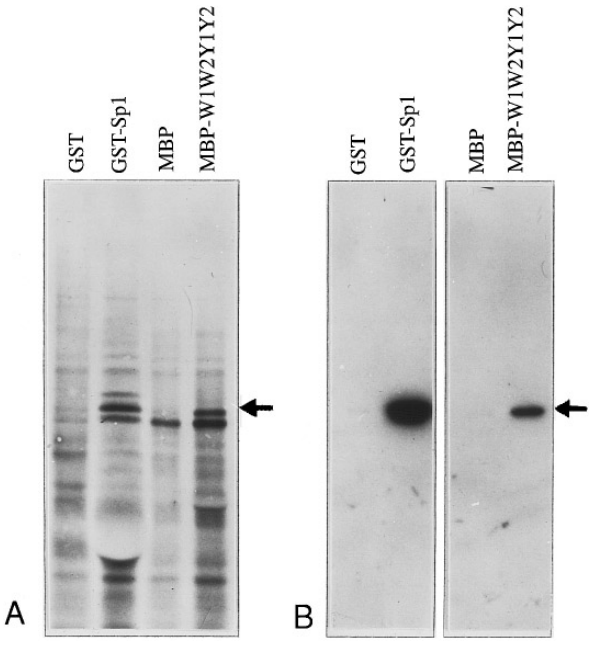

FIG. 2. Comparison of MBP–EBNA-LP fusion affinity precipitated p68 with the proteins associated with glutathione–S-transferase (GST) or GST–Sp1 fusions. (A) Beads coated with fusion proteins were used to affinity precipitate ^{35}S-labeled proteins from DG75 cell extracts. (B) Cellular proteins affinity precipitated by the fusion proteins were blotted and probed with an antibody which recognizes human HSP72/73 heat shock proteins.

A similar pair of proteins binds to the basal transcription factor Sp1

At approximately the same time as these experiments were being performed, a similar doublet banding pattern with the lower band often being more intense was also discovered in affinity precipitates with GST–Sp1 fusion proteins (John Horowitz, Duke University, personal communication). To determine if there was any correspondence between the protein species being detected, we first loaded ^{35}S-labeled proteins affinity precipitated with MBP–W1W2Y1Y2 and GST–Sp1 on the same gel to examine this relative mobility (Fig. 2A). The specifically interacting species precisely comigrated. Several experiments were performed where one fusion protein was used to attempt to deplete the cell lysate of the interacting protein before affinity precipitation with the other fusion protein. These did not work. It proved very difficult to deplete the lysate using repeated (up to six) consecutive precipitations with only one fusion. We suspected that the interacting species must not be a rare molecule but instead a relatively abundant cellular protein.

The proteins affinity precipitated by Sp1 and EBNA-LP are HSP72/73

Initially, nuclear lamins, about the same size as the p68, were considered but anti-lamin B and anti-lamin A/C antibodies did not react with either the Sp1 or the EBNA-LP affinity precipitated protein. An earlier report by Kaelin *et al.* (1991) had described a similar size doublet species interacting with Rb. Recently this doublet was identified as HSP72/73 (Nihei *et al.,* 1993). Antibodies against human HSP73 were tested by Western blotting and reacted with the protein affinity precipitated by Sp1 and EBNA-LP (Fig. 2B). Since no other bands appeared and the affinity precipitates with GST and MBP bacterial proteins contained no reactive species, we concluded that both Sp1 and EBNA-LP specifically interact with HSP proteins.

To map the location of domains within EBNA-LP responsible for the interaction with HSP72/73, fusion proteins composed of MBP fused to N-terminal and C-terminal deletions of EBNA-LP open reading frame were constructed (Fig. 3). The deletion sites corresponded to the exon structure (W1W2Y1Y2) of the EBNA-LP gene and hence deletion mutants were made which contained progressively fewer exons on the N or C terminus. The proteins with C-terminal deletions proved to be unstable in bacteria. After screening a panel of protein expressing clones and using short-time induction procedures, we were able to obtain pure W1W2 and W1 fusions in sufficient quantities to perform affinity assays. The W1W2Y1 fusion protein degraded too rapidly to be purified in sufficient quantities for these analyses. The ability of the mutants to work in affinity precipitation was analyzed using purified HSP72/73. Affinity precipitated proteins were immunoblotted and probed with anti-HSP73. Only fusion proteins containing the Y2 exon precipitated the HSP73 protein (Fig. 3C). The Y2 exon consists of only 34 amino acid residues, representing 31% of the total unique sequence of the protein.

EBNA-LP interacts with HSP73 *in vivo*

Identifying potential interacting species with fusion protein lures strongly suggests but does not prove that these proteins interact with EBNA-LP *in vivo* in EBV-infected cells. To ascertain whether HSP binds to EBNA-LP *in vivo,* extracts from whole EBV-positive lymphoblastoid cell lines BL36 and X50-7 were immunoprecipitated with monoclonal JF186 anti-EBNA-LP antibodies. Immunoprecipitates were blotted and probed for the presence of EBNA-LP and HSP73 (not shown). The HSP73 protein coimmunoprecipitated with EBNA-LP from both cell lines. Anti-EBNA-LP antibodies used with EBV-negative cell lysates (not shown) or irrelevant murine monoclonal antibodies used with EBNA-LP containing cell lysates failed to coprecipitate HSP73 (Fig. 4). In a cross-coprecipitation analysis, lysates of BL36 were first immunoprecipitated with EBNA-LP, HSP72/73, or Rb antisera and recovered products run on polyacrylamide gel. Each precipitate was then probed with anti-EBNA-LP and anti-HSP72/73 (Fig. 4). EBNA-LP immunoprecipitates contained HSP72/73. HSP72/73 immunoprecipitates contained EBNA-LP. Rb

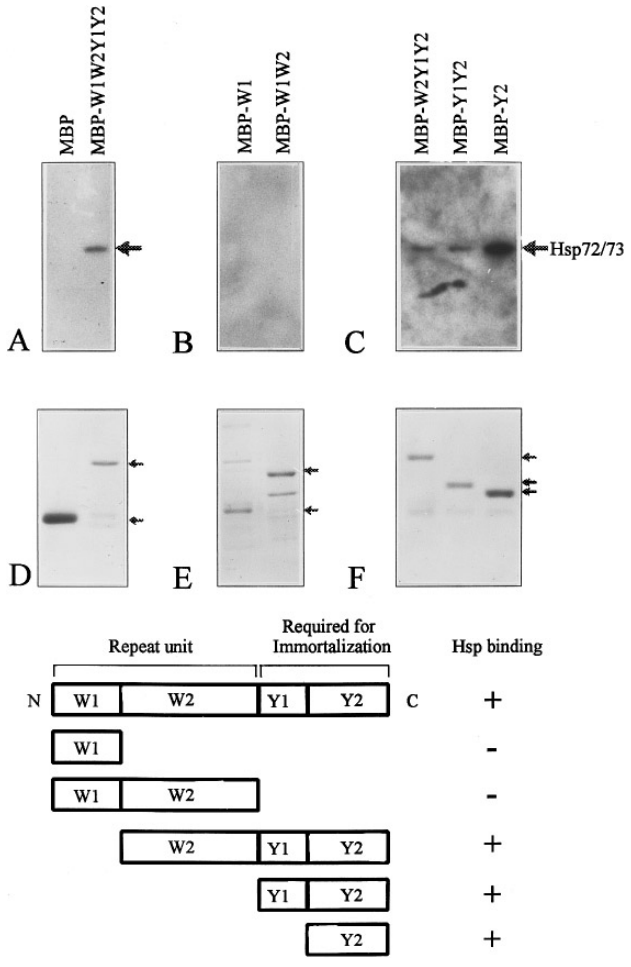

FIG. 3. Domains required for EBNA-LP binding to HSP. *In vitro* binding assay consisting of amylose bead coated with MBP–EBNA-LP fusions and purified soluble HSP73. (A) MBP and MBP fused to Wt EBNA-LP with all four exons, W1, W2, Y1, and Y2. (B) Fusions containing progressively fewer exons from the COOH end. (C) Fusions containing progressively fewer exons from the NH_2 end. A, B, and C were immunoblotted and probed with anti-HSP72/73. D, E, and F present the Coomassie-stained acrylamide gels of fusion proteins recovered from the loaded amylose beads. The diagram below the panel summarizes the results.

immunoprecipitates contained HSP72/73 but not EBNA-LP. The anti-Rb antibodies were used to coprecipitate HSP73 with Rb, a finding which has been reported previously and was therefore used here as a positive control. The EBNA-LP immunoprecipitate contained no detectable Rb (not shown) and the band near the position of EBNA-LP in the Rb immunoprecipitate is but an intense nonspecific band which appears as a containment in all the anti-Rb precipitates with this serum. Phosphorimages of the blots shown in Fig. 4 were analyzed to estimate the relative levels of protein present in the immunoprecipitates. The HSC immunoprecipitate contains 6.5% of the total HSC and 1.7% of the total EBNA-LP. Therefore, assuming that the immunoprecipitatable HSC is representative of the total, approximately 25% of the EBNA-LP in the cell is complexed with HSC. The EBNA-LP immunoprecipitate contains 7.2% of the total EBNA-LP and 0.1% of the HSC. Therefore, similarly, approximately 1.4% of the HSC in the cell is complexed with EBNA-LP.

EBNA-LP and Rb

EBNA-LP has been shown to colocalize in the nucleus with Rb by *in situ* immunofluorescence and form complexes with GST–Rb when that fusion protein is used as a lure. When MBP–EBNA-LP fusions were used as a lure we were unable to detect Rb in the affinity precipitates from DG75 cell lysates (Fig. 5A). Under identical conditions with the same lysates, GST–E1a, a fusion protein containing the adenovirus E1a protein sequences known to interact with Rb, was able to interact with Rb and draw it into affinity precipitates. Reversing the lures, and using GST–Rb to attract proteins from lysates of 293 cells (Ad 5 transformed human embryonic kidney cells expressing E1a) or X50-7 (EBV-positive LCL), produced similar results. GST–Rb interacted with the E1a in 293 cell lysates but not with EBNA-LP from X50-7 cell lysates. The presence of the potentially interacting proteins in the lysates was confirmed by immunoprecipitation with

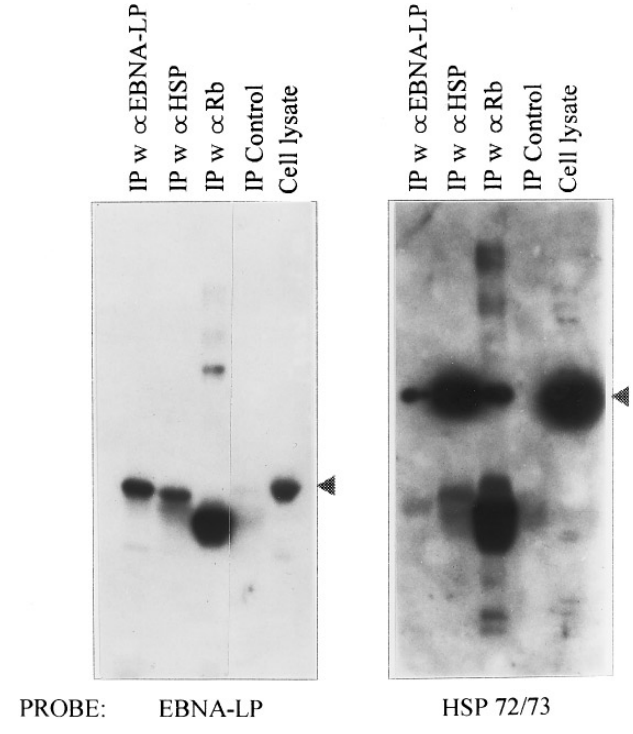

FIG. 4. Coimmunoprecipitation of EBNA-LP and HSP from EBV-positive BL36 cells. Proteins were initially immunoprecipitated with antisera specific for EBNA-LP, HSP, or Rb and then immunoblotted and probed with the same antisera. Rb immunoprecipitates contained two nonspecific bands which always appeared regardless of which antiserum was used in the immunoblots. Lysates from 10^7 cells were used for immunoprecipitations and a control representing 10^6 cells solubilized in electrophesis sample buffer was run to indicate the position of the specific protein bands.

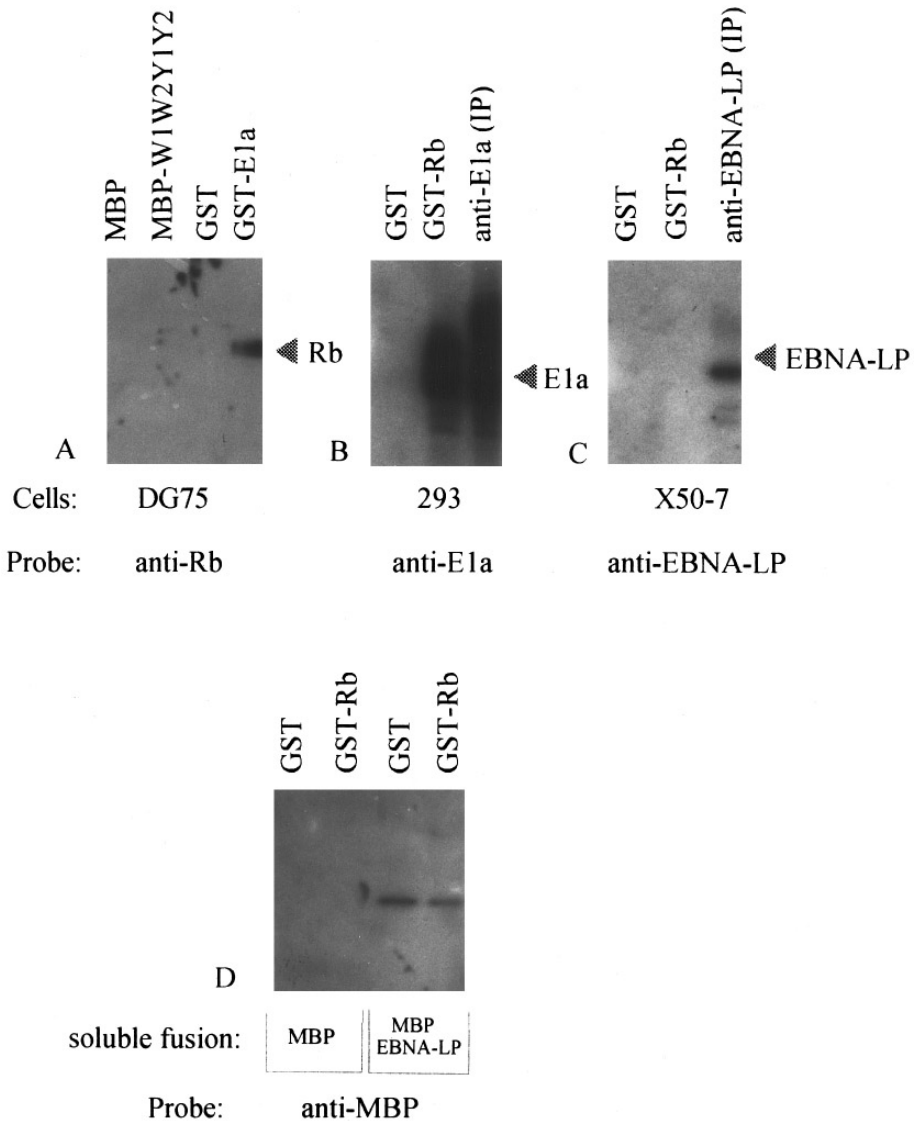

FIG. 5. Affinity precipitation of proteins by EBNA-LP, adenovirus, E1a, and Rb. (A) DG75 cell extracts were used with EBNA-LP and E1a fusion proteins as lures followed by anti-Rb immunoblotting. (B) Adenovirus E1a expressing 293 cells were extracted and used with GST Rb as the lure followed by anti-E1a immunoblotting. (C) EBNA-LP expressing X50-7 cells were used with GST–Rb as the lure followed by anti-EBNA-LP immunoblotting. In B and C the left lane represents an immunoprecipitation of the E1a or EBNA-LP target protein with specific antisera. (D) *In vitro* affinity interaction of MBP–EBNA-LP with GST–Rb with GST fusions attached to glutathione beads and MBP or MBP–EBNA-LP as the soluble target proteins.

the appropriate antibody (Figs. 5B and 5C). Directed MBP–EBNA-LP fusion protein and GST–Rb fusion protein interaction was performed in a cell-free buffer consisting of 20 m*M* Tris–Cl (pH 8.0), 100 m*M* NaCl, 0.5% NP-40 (Fig. 5D). While neither GST nor GST–Rb interacted with MBP, both GST and GST–Rb bound MBP–EBNA-LP. This indicated that there was some affinity of the GST–Rb for the MBP–EBNA-LP fusion that was dependent upon the EBNA-LP sequences; however, we were unable to show that the interaction detected required the presence of Rb sequences.

We have sequenced the EBNA-LP ORF being used in the above experiments and it is not mutated. The fusion proteins react with JF186, a monoclonal antibody that has its epitope in W1 where a putative Rb interaction domain is located. The positive controls demonstrate that other species are able to interact under the experimental conditions employed.

DISCUSSION

The EBNA-LP protein represents a unique viral nuclear oncogene. EBNA-LP is the first EBV gene product detected upon primary infection of resting B lymphocytes (Allday *et al.,* 1989). When the Y1 and Y2 exons are deleted from the viral genome, immortalization of B lympho-

cytes is debilitated but not entirely lost (Hammerschmidt and Sugden, 1989). Several lines of evidence now suggest that the protein plays a role in cell cycle control. Cell cycle transition delays in arrested mutant immortalized cells first suggested that EBNA-LP might function in G1 (Allan *et al.,* 1992). Recently, transfection of resting cells with EBNA2 and EBNA-LP expressing plasmids has suggested that the combination of these two proteins along with CD21 crosslinking to mimic virus attachment is able to lift resting cells out of G0 and into G1 (Sinclair *et al.,* 1994).

One therefore might suspect that a viral protein expressed early after infection, localized to the nucleus, and involved in cell cycle control might function by interacting with cellular growth control proteins. This has been a common theme among DNA tumor viruses that are otherwise unrelated in genome structure or sequence (Whyte *et al.,* 1988; Keen *et al.,* 1994; Kaelin *et al.,* 1991). The targets for their immortalization and transformation genes include the antioncogene cell cycle regulators Rb and p53. It was therefore an interesting, but not entirely unexpected, finding that EBV also deployed a protein (EBNA-LP) early after infection which colocalized and interacted with Rb and p53. Our affinity precipitation studies using ^{35}S-labeled cells and EBNA-LP fusion proteins did show a band in the vicinity of what might have been a p105 product under low-stringency wash conditions. Such experiments were complicated by high levels of background nonspecific binding. Attempts were made to improve the binding conditions to reduce the nonspecific binding and enhance the signal. Unfortunately, conditions were never found which reduced the high nonspecific background and maintained the loose association of a high-molecular-weight ^{35}S-labeled protein. Preliminary identification of the product as Rb was attempted using immunoblotting with Rb specific antisera. Affinity precipitated protein complexes even under the least stringent conditions did not contain Rb detectable by immunoblotting. Immunoprecipitation of *in vivo* expressed EBNA-LP blotted and probed for Rb coprecipitation was also negative as was immunoprecipitation of Rb blotted and probed for an associated EBNA-LP. We conclude that a specific association between Rb and EBNA-LP must be an extremely low-affinity phenomenon that occurs only in specially contrived circumstances not required to demonstrate other protein–protein interactions.

Consistently and specifically there was always a protein in affinity precipitates whose molecular weight was estimated to be approximately 68 kDa. This protein was shown to be HSP72/73 by immunoblotting affinity precipitates with anti-HSP72/73 specific antibodies. The same protein was also detected in affinity precipitates using GST–Sp1 as a lure and in coimmunoprecipitates with EBNA-LP antibodies and Rb antibodies from cell extracts. The heat shock protein family has been implicated in several important physiological processes. They function as molecular chaperones, transporting peptides and immature proteins to cellular organelles and assisting in protein folding to ensure proper conformation and subunit organization (Palleros *et al.,* 1991; Hightower, 1991; Flynn *et al.,* 1991). In the nucleus both Rb and p53 interact with HSPs and for p53 frequently the association is with the mutant forms of this protein (Hinds *et al.,* 1987). It is tempting to speculate that conformational changes, perhaps influenced by cell-cycle-dependent phosphorylation, accompany protein–protein interaction during the functioning of cell cycle regulators like Rb (Chen *et al.,* 1989). HSPs may be involved in stabilizing complex formation and/or in recharging spent regulators by facilitating their return to an active conformation following dephosphorylation. In this way EBNA-LP may be similarly involved in transient complexes and undergo cell-cycle-dependent conformational changes requiring or at least influenced by HSPs.

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